Mutation Rate Analysis to Study Aminoacylation Deficiencies

Undergraduate Research Thesis

Presented in partial fulfillment of the requirements for graduation with *honors research distinction* in Microbiology in the undergraduate colleges of The Ohio State University

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Abstract:

In recent years, numerous defects in tRNA biogenesis and related processes, including tRNA aminoacylation, have been linked to human disease. As direct mediators of translational fidelity, aminoacyl-tRNA synthetases (aaRS) are responsible for the correct pairing of an amino acid with its cognate tRNA. Mispaired aminoacyl-tRNA (aa-tRNA) species occur due to a lack of amino acid discrimination within the aaRS active site. These misacylation events make tRNA proofreading and editing mechanisms essential to maintaining the accuracy of translation. Historically, misincorporation of Tyr into the proteome at Phe codons has been considered a major contributor to an overall decrease in cell viability.

Looking at the accumulation of mutations over time is useful for identifying factors that contribute to disease progression, but does not provide much insight into the source of mutations. It is possible that mutation rates are affected by factors other than proteome fidelity in this model. To address this question, a yeast-based mutation rate analysis will be performed, thereby precluding contributing factors of the tumor microenvironment. We hypothesize that changes in proteome fidelity caused by aaRS mediated mistranslation lead to dramatic genomic alterations through an increase in mutation rate. A mutation fluctuation assay to monitor cell survival, which reflects the frequency of inactivation of the *CAN1* locus, will be performed. CAN1 is responsible for importing canavanine, a toxic analog of arginine. Data obtained from the mutation rate analysis will provide insight into the direct contribution of aaRS mediated mistranslation to genome stability. If these initial experiments are successful, our results could be further applied to human cell lines using the endogenous yeast enzyme.

Introduction:

Research in recent years has shown a link between human disease and defects in tRNA biogenesis and related processes, including tRNA aminoacylation. Aminoacyl-tRNA synthetases (aaRS) are the mediating factors in translational fidelity that coordinate the correct pairing of an amino acid with its cognate tRNA. A lack of discrimination between amino acids within the aaRS active site typically causes mispaired aminoacyl-tRNA (aa-tRNA) species. As a result of these misacylation events, tRNA proofreading and editing mechanisms are essential to maintaining the accuracy of translation. Without these mechanisms, translational fidelity can be drastically decreased. The two main mechanisms by which translational fidelity is maintained within the cell are through accurate synthesis of aminoacyl-tRNAs and efficient selection of tRNAs during decoding at the ribosome. Proofreading of misacylated tRNAs by aaRSs is a vital component of quality control. In *Saccharomyces cerevisiae*, the β-subunits of the heterotetrimeric phenylalanyl-tRNA synthetase (PheRS) is the location where editing of misacylated Tyr-tRNA^{Phe} occurs. When PheRS editing activity is eliminated, there are often severe growth defects in the presence of high concentrations of the near-cognate amino acid, tyrosine (Tyr). Traditionally, misincorporation of Tyr into the proteome at phenylalanine (Phe) codons has been considered a major contributor to an overall decrease in cell viability. Despite the vast research supporting the relationship between aa-tRNA editing and mistranslation, less research has been dedicated towards establishing the effects of aa-tRNA editing on the regulation of critical cellular processes and maintenance of genome stability.

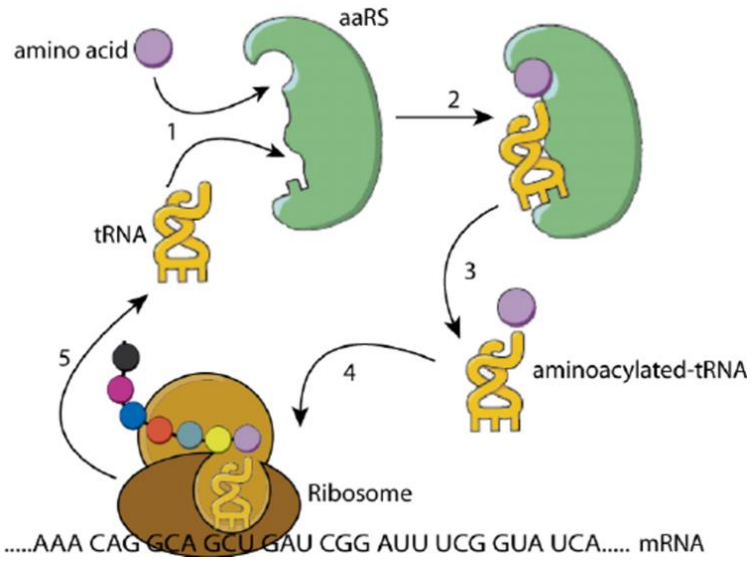


Figure 1: Aminoacyl-tRNA synthetase role in the cell.

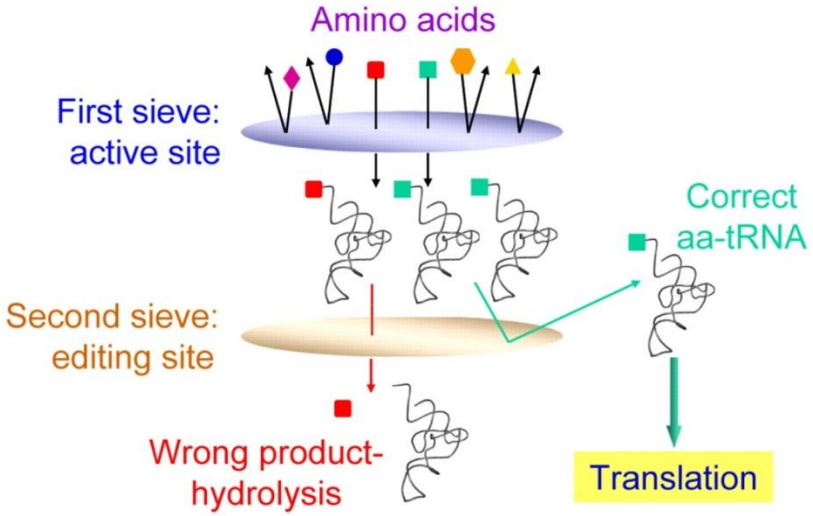


Figure 2: Editing ability of aaRS enzymes.

Intracellular amino acid pools are monitored by the general amino acid control (GAAC) pathway through tracking the accumulation of deacylated tRNAs. The aaRSs coordinate the pairing of cognate amino acids and deacylated tRNAs, which maintains specificity via quality control pathways. It has been shown in *Saccharomyces cerevisiae* that aaRS quality control is

essential for maintaining cellular viability during nutritional stress (1). The authors demonstrated that if aaRS quality control was impaired, then the levels of deacylated tRNAs were altered, and efficient activation of the GAAC and target of rapamycin (TOR) pathways would be prevented. After quality control was impaired, they found a significant accumulation of Tyr-tRNA^{Phe} and 6-8% Tyr misincorporation at Phe codons, which were also observed *in vivo*. The primary conclusion was that, when presented with certain abnormal stress conditions, the aaRS quality control primarily functions to ensure efficient activation of nutritional stress responses rather than to prevent mistranslation events.

These recent studies have suggested that increased error rates in protein synthesis are an important facet of stress responses during amino acid limitation (2). However, disruption of the proteome has been shown to directly cause tumorigenesis and increase susceptibility to pro-tumorigenic stress (2). Carcinogenesis at its very basic level is a collection of mutations that permit uncontrolled cell proliferation. For the cancer to progress, it must adapt new mechanisms to evade the host immune system and establish sub-clonal populations with specific mutations that permit metastatic growth.

Looking at the accumulation of mutations over time is useful for identifying factors that contribute to disease progression, but does not provide much insight into the source of mutations. It is possible that mutation rates are affected by factors other than proteome fidelity in this model. To address this question, a yeast-based mutation rate analysis will be performed, thereby precluding contributing factors of the tumor microenvironment. We hypothesize that changes in proteome fidelity caused by aaRS mediated mistranslation will lead to dramatic genomic alterations through an increase in mutation rate. Mutation rate will be monitored by measuring cell survival, which reflects the frequency of inactivation of the *CAN1* locus, as described

previously (3). CAN1 is responsible for importing canavanine, a toxic analog of arginine. After incorporation of canavanine into proteins instead of arginine, the yeast cells are killed by the increased toxicity. Data obtained from the mutation rate analysis provides insight into the direct contribution of aaRS mediated mistranslation to genome stability. If these initial experiments are successful, our results could be further applied to human cell lines using the endogenous yeast enzyme.

Methods and Results:

Mutation Rate

Mutation Rate Analysis by a Fluctuation Assay:

In evolution, the mutation rate is a very important parameter to consider. The mutation rate controls the speed that beneficial adaptations can be introduced into populations. However, it is often difficult to estimate the mutation across an entire genome per a single generation. Traditional methods to study mutation rates take a three-step approach by determining the mutation rate to a particular phenotype, determining the associated nucleotide base pair mutation rate from the phenotypic rate, and using these rates to estimate the mutation rate for the entire genome. Using this method, the phenotypic mutation rates to canavanine, for instance, were found to be 1.52×10^{-7} (4).

Salvador Luria and Max Delbruck first used a mutation rate assay in 1943 (5). They looked at both the mutation rate (number of mutation events per cell division) and the mutant frequency (the number of mutant cells per culture). However, the difficulties with such

experiments are that a mutation rate can be the same but with different mutant frequencies. For instance, if there are two mutation events in two different cultures then by definition, the mutation rate is the same. But, if these mutation events happen in different generations, then for the cultures where the mutation events occurred in an earlier generation the mutant frequency is higher. The traditional Luria-Delbruck distribution measures the number of mutant cells per culture. On the contrary, a Poisson distribution measures the number of mutation events per culture, thus allowing a more accurate mutation rate to be determined. The distribution of the number of mutant cells per culture is measured along with the number of cells per culture (N). By examining the average number of mutation events per culture (m) the mutation rate can be evaluated ($\mu = m/N$).

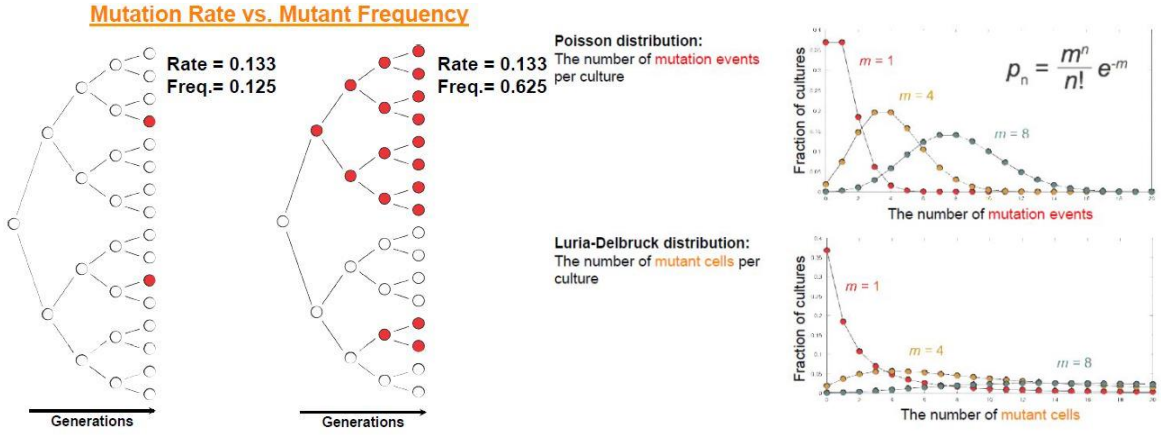


Figure 3: A Luria-Delbruck distribution plots the number of mutant cells per culture (bottom right), however, a Poisson distribution plots the number of mutation events per culture (top right). The image on the left demonstrates the importance of this distinction because each of the three cultures has two mutation events over four generations. However, each of the generational schemes has a different number of mutant cells and thus a different mutant frequency. But the mutation rate in each is the same.

Determining the average number of mutation events per culture was done by performing an assay of many cultures on canavanine. Plating 1000 canavanine-sensitive cells into 96-well plates allows for this determination in a quantifiable nature. The method for enumerating the mutation rate is called the P_0 method. This method uses the fact that they only way to get zero mutant cells is if there were zero mutation events. The simplest way to estimate the expected number of mutants that occur in each culture (m) is from the fraction of cultures with zero mutants (e^{-m}).

$$P_n = m^n/n! \times e^{-m}$$

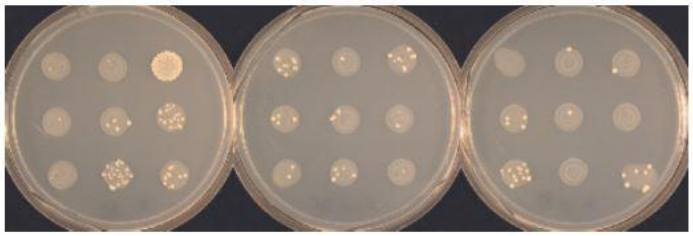
$$P_0 = m^0/0! \times e^{-m}$$

$$P_0 = e^{-m}$$

$$m = -\ln(P_0)$$

Therefore, if a known average number of total colonies are spot-plated onto canavanine, then the number of spots that have no growth (which have the mutation that imports canavanine into the proteins) are taken out of the total number of spots plated (P_0 = number of spots with no growth/total number of spots plated). The N value is the average number of cells plated per spot (or that have grown in the 96 well plates). Thus, the mutation rate is defined as (4):

$$\mu = -\ln(P_0)/N$$



The P0 method
The only way to get 0 mutant cells is if you had 0 mutation events

$$p_n = \frac{m^n}{n!} e^{-m}$$

$$p_0 = \frac{m^0}{0!} e^{-m}$$

$$p_0 = e^{-m}$$

Method 1 for calculating *m*

$$m = -\ln(p_0)$$

Figure 4: P_0 method for calculating the mutation rate.

Canavanine mutants are useful to study due to the physiology behind canavanine import. As it is a toxic analog of arginine and regulated by the arginine transporter, taking up canavanine is detrimental to the cells. Loss-of-function mutants of the transporter, encoded by the *CAN1* gene, can be selected for by supplementing media with canavanine. The mutation events themselves occur while the cells are growing in the 96 well plates. Thus, the canavanine-sensitive cells divide and, through mutation, produce canavanine-resistant progeny. Many mutations that occur in cells can be detected and fixed; however, under stress conditions these proofreading mechanisms are less efficient in preventing mutation events. In fact, cells are programmed to overlook mutations that might be beneficial to cell survival under stress conditions.

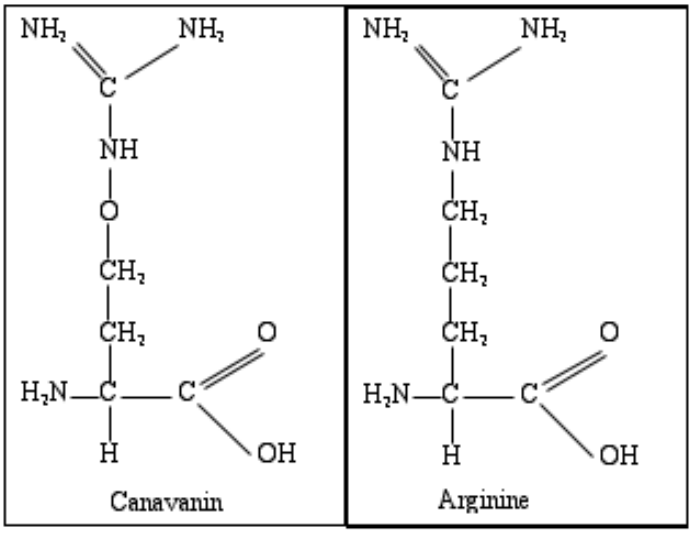


Figure 5: Canavanine is a molecular analog of arginine.

Strain Construction:

To insert the *CAN*1 gene into yeast a CORE cassette was used. The CORE (COunterterselectable REporter) cassette allows the organism that contains this sequence to be able to grow on plates lacking uracil (URA^-) as it contains the $URA3^+$ gene to synthesize uracil. The CORE cassette also contains an antibiotic resistance marker to G418 which is used to select for mutant yeast colonies. This cassette, contained in an *E. coli* plasmid (pCORE), was extracted by plasmid miniprep and amplified by PCR. It was determined that a large quantity of CORE template was needed for subsequent transformation.

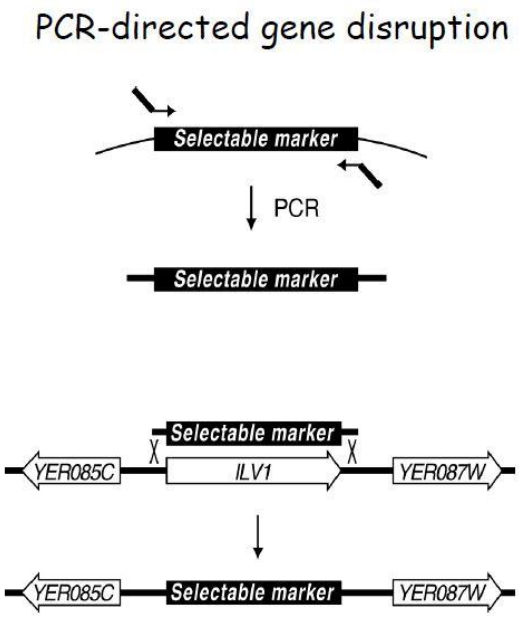


Figure 6: CORE cassette transformation by homologous recombination.

Colonies of wild type *S. cerevisiae* (NR1), and *S. cerevisiae* with a mutation in the *FRS*1 gene (NR2), which makes the strain defective in PheRS editing activity, were streaked out and grown in liquid media overnight. The wild type and mutant yeast strains were transformed with the CORE cassette using a transformation method called the *delitto perfetto* approach (6). This approach uses a double stranded break (DSB) in the host genomic DNA to homologously recombine the CORE sequence into the host. The CORE cassette is flanked by two sequences that are homologous to two adjacent sequences in the host genome. When the CORE cassette is transformed into yeast, the two previously adjacent sequences are then separated from each other by the CORE sequence.

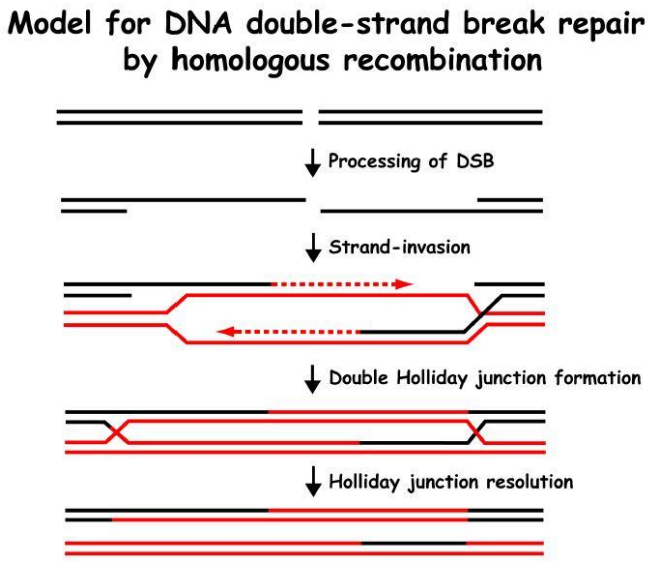


Figure 7: The delitto perfetto approach to in vivo mutagenesis involves creating a double stranded break (DSB) that is repaired by homologous recombination with the mutant gene (right). This is contrasted to the traditional approach where a gene is disrupted in one step.

These transformed colonies were plated on YPDA media (YPD with adenine supplemented in the media). To confirm the integration, the candidate transformants were patch plated onto SD media lacking uracil (SD-URA). Due to the presence of the CORE cassette, confirmed integrants were able to grow on this media. Before confirming integrant growth with G418 present in the media, the integrants were grown overnight in SD-URA. They were then streaked onto YPD and YPD+G418. Following growth on these plates, DNA from both NR1 and NR2 containing the CORE cassette was extracted. The integrations were confirmed at the genetic level by PCR using primers that amplified the CORE sequence. After confirming the integration, glycerol stocks were made to store for further use.

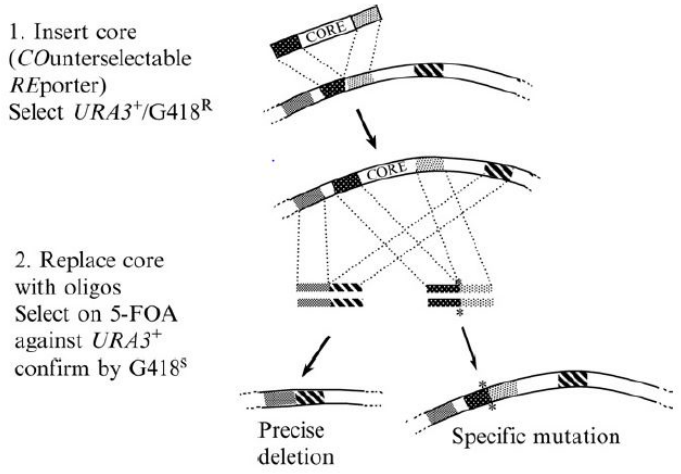


Figure 8: Delitto Perfetto overall scheme.

A gBlocks gene fragment from Integrated DNA Technologies was obtained containing the *CAN*1 gene. This gene fragment was amplified by PCR and transformed into NR1 and NR2 strains using the same *delitto perfetto* protocol as the insertion of the CORE cassette (6). This transformation requires homologous recombination of the two sequences on either end of the CORE cassette with the similar sequences of the gBlocks gene fragment. However, the gBlocks template contains the gene of interest, here the *CAN*1 gene that imports canavanine and kills the cells. Thus, through transformation by this method a specific mutation in the gene of interest can be efficiently inserted the host genome *in vivo* by homologous recombination.

The candidate integrants were plated on YPDA immediately following transformation. These colonies were then replica plated onto 5-fluoroorotic acid (5-FOA) media. If the *URA*3 gene is present in organisms that are plated on 5-FOA, then the cells convert the 5-FOA into 5-fluorouracil, which is toxic. Therefore, if the integration was successful then the integrants will grow on 5-FOA. However, if the integration of the target gene was not successful, then there would no growth on these plates. Thus, these 5-FOA plates were saved as the master plates.

To confirm the integration of the *CAN*1 locus, the master 5-FOA plates were replica plated onto canavanine, 5-FOA, YPD+G418, and YPD. By replica plating in this manner, confirmed integrants should not grow on canavanine and G418, but grow on 5-FOA and YPD. Four NR1 and four NR2 strains were isolated and restreaked on canavanine to confirm their identity. These cultures were labeled NR1/NR2 CAN1A-D and were saved as glycerol stocks. From the overnight cultures the yeast cells were pelleted before the genomic DNA was extracted and amplified by PCR. The PCR confirmed the integration and the retention of the *frs1-1* mutation.

Mutation Rate Determination:

The mutation rate assay was performed by streaking NR1 and NR2 CAN1A onto YPD for approximately three days. A single culture of each strain was inoculated into SDMM and grown overnight. The concentration of each overnight culture was determined and subsequently diluted in order to transfer 1000 cells into 54 wells of a 96-well plate. The cells were diluted with either SDMM or 1:400 Phe:Tyr SDMM and allowed to grow for approximately 36 hours. The average concentration of each well was determined (N). The cells from each well with potential mutant yeast cells were plated onto canavanine. Nine spots were plated on each plate and allowed to grow for 48 hours.

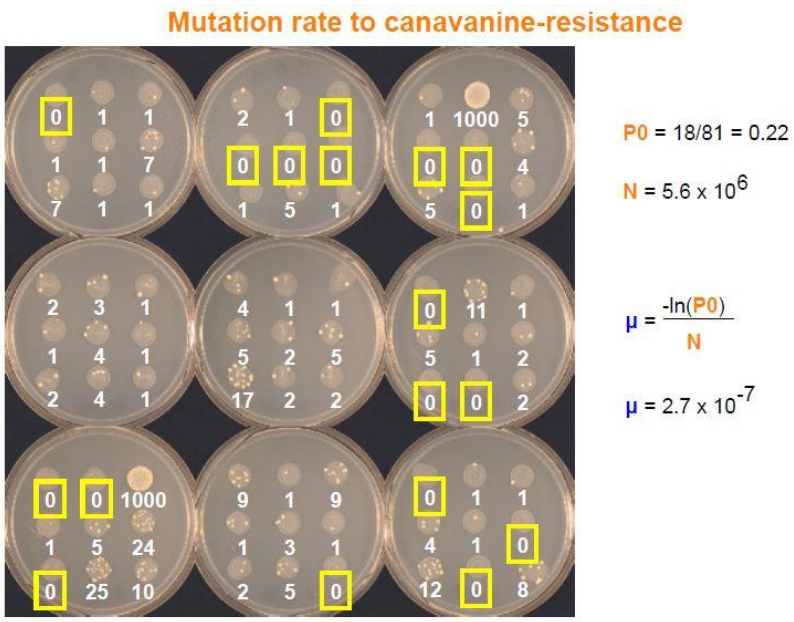


Figure 9: Cells that grow on canavanine have an inactivated canavanine importer (encoded by CAN1). The spots that had no growth were quantified and the frequency of maintained CAN1 function was used to evaluate the mutation rate.

The frequency that spots had no growth was recorded (P_0) and used to determine the mutation rate. The results of the mutation rate are as follows:

Media Condition	Strain	Average Mutation Rate	Number of Replicates (n)
SDMM	NR1	2.98E-8	6
	NR2	1.86E-8	5
1:400 Phe:Tyr SDMM	NR1	1.54E-6	4
	NR2	7.97E-6	2

Table 1: Mutation rate of NR1 and NR2 under minimal media and minimal media with high Tyr content.

The mutation rate in minimal media was two orders of magnitude less than in Tyr-rich media. Comparatively, the NR1 and NR2 strains had a relatively similar mutation rate under the minimal media conditions. Since there is no unusual stress conditions on these cells, the strain lacking PheRS editing activity, NR2, has a mutation rate similar to that of yeast with functional editing. On the other hand, the mutation rate under high Tyr conditions is significantly higher for the wild type and mutant strains. However, the mutation rate for the editing deficient strain is approximately half an order of magnitude higher than wild type yeast.

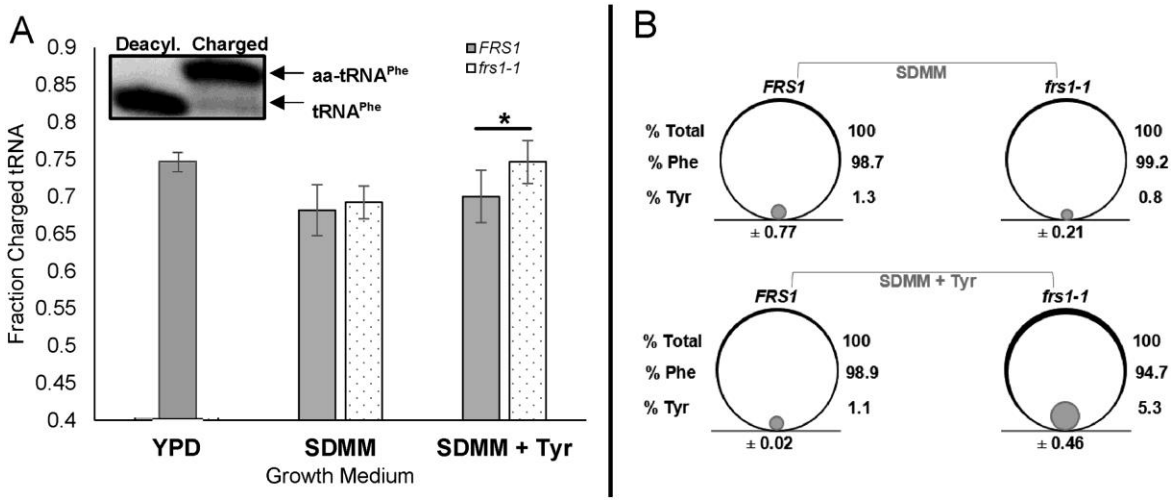


Figure 10: Isoacceptor Specific Aminoacylation Profiling (ISAP) shows the amount of Phe and Tyr that are bound to PheRS. (A). The levels of acylated and deacylated tRNA^{Phe} are shown in both minimal media and high Tyr conditions. (B). The percentages of Phe and Tyr bound to tRNA^{Phe} were measured under minimal media and high Tyr conditions. Under the high Tyr stress more Tyr was bound to tRNA^{Phe} than under no stress.

Thus, the fluctuation assay indicates that NR2 has a higher mutation rate than NR1 when grown in high Tyr conditions. The higher mutation rate exhibited by NR2 means that there were more NR2 colonies that had an inactivation of *CAN*1 than in NR1. Since NR2 lacks PheRS

editing activity, this likely means that there was misincorporation of Tyr that led to mutations in the *CAN*1 locus, thereby inactivating it. Without the editing activity of PheRS, the NR2 cells were more susceptible to mutation under high Tyr stress and thus a higher mutation rate was observed.

aaRS Mutations

Misacylation events are directly linked to mutations. Therefore, tRNA proofreading mechanisms are critical in maintaining accuracy in translation. Compared to the research on the role of aa-tRNA editing in the context of mistranslation, the role of aa-tRNA editing in the regulation of critical cellular processes is understudied. The main hypothesis behind this research is that deficiencies in the aa-tRNA fidelity can cause significant changes on cellular processes beyond the ascribed role of mistranslation. Some research has shown that various stress signaling pathways and adaptive translation strategies are linked together into a comprehensive stress network system. This system incorporates environmental information and the overall aminoacylation status of the intracellular tRNA pool. The hypothesis is that there is an increase in misacylated tRNA, due to a lack of aa-tRNA editing, that decreases the cell's ability to accurately sense nutritional stress leading to reduced stress tolerance and cell viability. It has been shown that deregulation of aaRSs is linked to various pathologies including neurodegeneration, immune modulation, and cancer (7). The most frequently observed deregulation event is the overexpression of aaRSs in tumor tissues (8). Overexpression of aaRSs can lead to competition for limited tRNA substrates, causing an accumulation in non-cognate

tRNA acylation and thus an increase in the biologically available aa-tRNA species (9). Furthermore, misacylated aa-tRNA species due to aaRS overexpression can result in protein misfolding if the aa-tRNAs are recognized and incorporated into proteins by the ribosome and other translation machinery. This is similar to the case of overexpressed proteins, which can often be misfolded, and contribute to activation of the cell's protein stress responses.

Recently, defects in the nuclear genes encoding mitochondrial tRNA synthetases have become increasingly linked to several different pediatric onset disorders that primarily affect the central nervous system (10). The individuals suffering from these diseases often have neurologic difficulties through compound heterozygous mutations that present with system-specific phenotypes. The prevailing hypothesis is that these defects in the mitochondrial tRNA synthetases produce clinical phenotypes through decreased mitochondrial translation. One specific example involves *FARS*2 which encodes mitochondrial PheRS (mtPheRS). Mutations in this gene have been reported in six cases of an infantile, lethal disease with refractory epilepsy and progressive myoclonus. By observing the protein structure of the deficient polypeptides, it was concluded that mutations affecting the tertiary structure of mtPheRS lead to decreased functional capabilities. Defects in mtPheRS likely would result in changes in the expression of 13 mitochondrially-encoded peptides, of which include subunits of the respiratory chain (11).

It was shown, in conjunction with other similar findings (12) that there was a distinct genetic association with the attenuated phenotype. In other words, mistranslation is linked to mutation which can lead to disease. Therefore, it is suggested that there is a distinct neuropathologic process related to mutations in mtPheRS function. The study sheds more light on the variation in the phenotype of diseases associated with *FARS*2 mutations and also validates the use of

increased genetic testing to assess the nature of severe refractory epilepsy and developmental delay.

Mutations in tRNA synthetases are often heritable gene defects that can be dominant or recessive disease-causing mutations (13). However, the mutations that cause the diseases do not all necessarily affect the aminoacylation activity or general enzyme stability. The tRNA synthetases arose early in evolution and it is theorized that these enzymes are involved in immune function when they are secreted as procytokines to operate in immune amplification or angiogenesis (14). Additionally, tRNA synthetases control specific signaling pathways by forming multiprotein complexes with other synthetases or regulatory factors in cells. Thus, there are several different ways in which aaRSs can be disrupted to cause disease.

One specific example of a disease involving a heritable mutation in glycyl-tRNA synthetase (GlyRS) and tyrosyl-tRNA synthetase (TyrRS) is Charcot-Marie-Tooth (CMT) disease. CMT is the most frequently inherited disease of the peripheral nervous system and is caused by axonal deymyelination or decreased motor or sensory nerve responses. The mutations causing the disease are dominant but do not affect aminoacylation. GlyRS mutations were shown to affect synthetase dimer formation through a change in the surfaces on the dimer interface (15). On the other hand, TyrRS mutations that cause a missense or deletion mutation in the enzyme lead to the induction of axonal degeneration in differentiating neuronal cells (16). Thus, it was concluded that synthetases have a role in development and homeostasis, in addition to their role in protein translation. Mutations that affect these additional functions lead to disease.

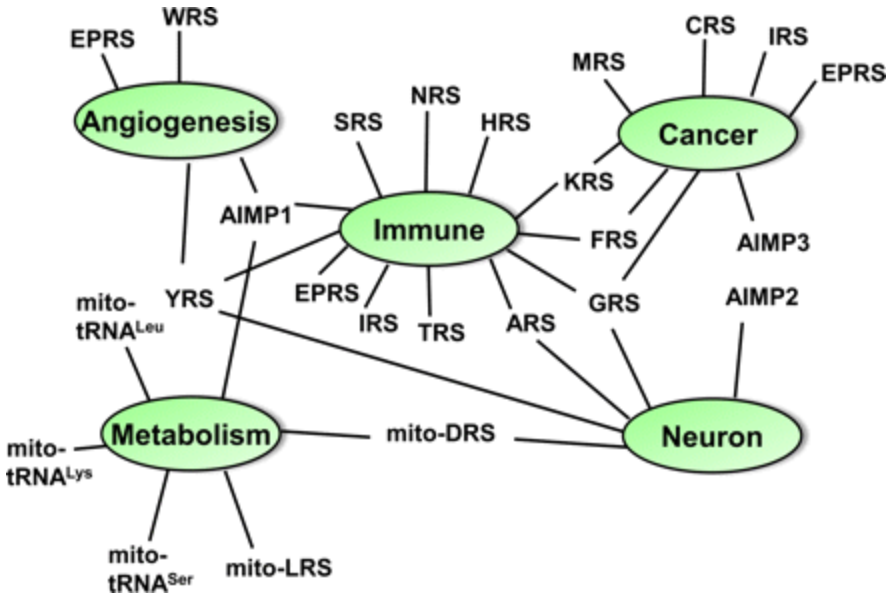


Figure 11: Map showing the connection of aaRSs to types of human diseases.

It is clear that aaRSs are involved in recognition of amino acids, tRNA and ATP, dynamic cellular localization, and a variety of protein-protein interactions. Therefore, aaRSs must play a key role in a cell's molecular response to quickly and efficiently maintain homeostasis at the cellular level by adapting to an assortment of cellular stresses. In the continuing battle to develop potential therapeutic drugs to fight cancer and other diseases, the study of aaRSs regulatory complexes provides new perspectives on the numerous translational factors that control disease.

Editing of misaminoacylated tRNA and the amino acid stress response

In response to nutritional stressors, such as amino acid stress, an increase in mistranslation can result from a decrease in the overall fidelity of the aa-tRNA pool. The mutation rate fluctuation assay demonstrated that under high Tyr conditions in the environment, yeast lacking

PheRS editing activity has a higher mutation rate than strains with editing activity. The likely cause of this is misincorporation of Tyr by PheRS resulting in mutant proteins. In this case, the nutritional stress is a relative lack of Phe in the media, which means that an editing deficient PheRS strain is more likely to incorporate Tyr instead of Phe into proteins. However, misincorporating Tyr instead of Phe results in a decrease of Tyr in the amino acid pool. Over time, this could create an additional amino acid deficiency in the pool and further decrease the aa-tRNA pool fidelity.

In eukaryotes, amino acid starvation activates the protein kinase GCN2, which leads to changes in gene expression and protein synthesis as part of a global stress response (17). The signal for GCN2 activation is deacylated tRNA, which accumulates when tRNA aminoacylation is limited either through lack of substrates or inhibition of synthesis. Pairing of amino acids and deacylated tRNAs is catalyzed by aaRS, which maintain cognate substrate specificity via quality control pathways. For example, as it has been previously discussed, PheRS maintains specificity via a specialized editing pathway that targets the non-cognate aminoacylation product Tyr-$tRNA^{Phe}$. While the primary role of aaRS editing is to prevent mistranslation, it was demonstrated that editing of non-cognate aminoacyl-tRNA is also required for proper detection of amino acid starvation by GCN2. Ablation of PheRS editing led to the accumulation of Tyr-$tRNA^{Phe}$ but not deacylated $tRNA^{Phe}$ during amino acid starvation, limiting GCN2 kinase activity and suppressing GCN4-dependent gene expression. While the PheRS-editing ablated strain grew 50% slower and displayed a 27-fold increase in the rate of mistranslation of Phe codons as Tyr compared to wild type, the increase in mistranslation was not sufficient to activate an unfolded protein stress response (17). Taken together, these findings show that during amino acid starvation the primary role of aaRS quality control is to help the cell mount an effective stress

response, independent of the role of editing in maintaining translational accuracy. This research provides more evidence for the relationship between aaRS and cellular homeostasis as hypothesized by researchers looking at disease.

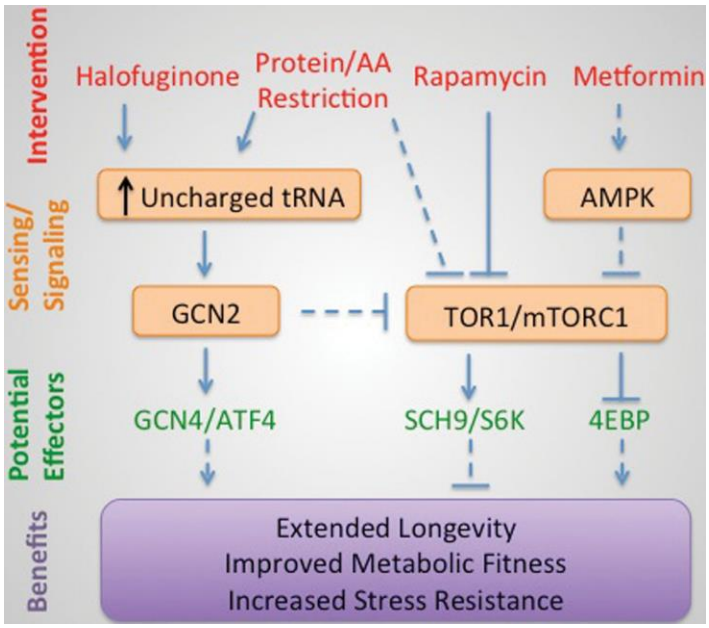


Figure 12: General Amino Acid Control Pathway.

tRNA is the most abundant non-coding RNA, comprising 4-10% of the total intracellular RNA pool (18,19). Regulation of the biosynthesis, modification, and degradation of tRNA directly impact translation and extend the role of tRNA beyond the canonical function of decoding genetic information (17). aaRS proofreading mechanisms prevent misaminoacylated tRNA accumulation through hydrolysis of misactivated aminoacyl adenylates (pre-transfer editing) and hydrolysis of misaminoacylated aa-tRNA (post-transfer editing). While aa-tRNA proofreading has been ascribed a role in minimizing mistranslation, far less is known about how this conserved step in translation quality control might regulate other cellular processes (17).

The aminoacylation status of the intracellular tRNA pool is a primary signal for cellular stress response pathways in both bacteria and eukaryotes. In eukaryotes, the general amino acid

control (GAAC) pathway alters the translational status of the cell as a means to regulate the transcriptional response to amino acid starvation (20, 21). The GAAC indirectly monitors intracellular amino acid pools through surveillance of deacylated tRNA accumulation by the protein kinase GCN2p. Activation of the GAAC occurs when deacylated tRNA binds to a region of GCN2p homologous to histidyl-tRNA synthetase thereby disrupting interdomain interactions (17). The activated kinase phosphorylates residue S51 on eIF2a which competitively inhibits the turnover of eIF2-GDP to eIF2-GTP, thereby reducing the pool of active ternary complex (TC) available for translation initiation. Reduced levels of TC result in a decrease in global translation, but an increase in the expression of the transcription factor GCN4p, which is responsible for the transcriptional reprogramming of cells in response to amino acid deprivation (17). Similarly in bacteria, accumulation of deacylated tRNA activates the stringent response, which like the GAAC leads to changes in gene expression at the levels of both transcription and translation (22). When deacylated tRNA enters the A-site of the bacterial ribosome, the enzyme RelA activates the stringent response through the production of the second messenger ppGpp(p). ppGpp(p) directly affects the translational and transcriptional status of the cell in response to nutrient stress (22). In bacteria, loss of aaRS-mediated tRNA quality control limits deacylated tRNA accumulation during amino acid starvation, which in turn suppresses the stringent response and prevents an efficient stress response (17). The loss of aaRS editing of misaminoacylated tRNAs also leads to significant activation of protein stress responses, presumably in response to increased accumulation of misfolded proteins resulting from mistranslation. While these studies identified a role for translational quality control in determining the sensitivity and specificity of nutritional stress responses, whether this function was dependent on mistranslation remained unclear. To investigate the mechanisms by which

aaRS-mediated editing of misaminoacylated tRNAs help to regulate cellular stress responses, we have now used *Saccharomyces cerevisiae* to determine whether eukaryotic amino acid starvation sensing is also linked to translation quality control (17). As with the bacterial stringent response, we found that accurate monitoring of amino acid starvation by the yeast GAAC is dependent on aaRS editing translation quality control to ensure proper accumulation of deacylated tRNA species. In the absence of editing under these growth conditions, misaminoacylated tRNAs accumulated to significant levels but yeast protein stress responses were not activated. Overall, these data reveal a critical function for aaRS-editing in stress responses that is independent of their role in preventing mistranslation.

Furthermore, PheRS editing activity is required for optimal growth during amino acid stress. It was shown that a deficiency in aa-tRNA editing suppresses the activation of the GAAC (17). Tyr-tRNA^{Phe} accumulation does not lead to activation of protein stress responses. These data indicate that despite a significant increase in the rate of mistranslation during growth under amino acid limitation, the absence of PheRS QC does not result in a sufficient increase in protein misfolding to activate the corresponding stress responses.

Accumulation of misaminoacylated tRNA^{Phe} prevents proper activation of the GAAC pathway:

Yeast cells assess the status of intracellular amino acid pools indirectly by monitoring tRNA aminoacylation levels via the GAAC pathway. The GAAC response uses deacylated tRNAs as signaling molecules to indicate stress, which provides a means to both accurately gauge the translational capacity of the cell and detect changes in the growth environment (17). The GAAC response is activated by various stressors and here we demonstrated that one of these, amino acid starvation, can only be accurately sensed by the cell when non-cognate aa-tRNA editing is

active. In the absence of editing, misaminoacylated tRNA accumulates, and the cell's ability to sensitively respond to nutritional stress is disrupted. Because this ability depends on deacylated tRNA levels accurately reflecting the availability of the corresponding cognate amino acids, the net result is that PheRS-QC deficient cells grow more slowly than wild type during amino acid starvation. This result is presumably due to their inability to adequately increase Phe synthesis via the GAAC pathway, as exemplified by suppression of ARO3 transcription. ARO3 encodes DAHP synthase, which catalyzes the first step in aromatic amino acid biosynthesis in yeast. The insensitivity of the PheRS-QC deficient strain to cognate amino acid starvation was circumvented either by supplementation with exogenous Phe or constitutive induction of GCN2 or GCN4, further supporting the role of misaminoacylated-tRNA editing in tuning the sensitivity of the GAAC response. A role for PheRS QC in activation of amino acid starvation responses was first reported for *E. coli* grown in the presence of the cytotoxic non-protein amino acid *meta*-Tyr, where the absence of misacylated tRNA editing delayed transcription of the gene encoding chorismate mutase (17). When combined with previous findings, our data show that PheRS QC plays similar roles in bacteria and in eukaryotes, albeit via regulation of different steps in aromatic amino acid biosynthesis and in response to non-protein versus non-cognate amino acids, respectively.

Codon specific misincorporation of Tyr-tRNA^{Phe} is not cytotoxic:

Errors in protein synthesis arise from a variety of causes including inaccurate transcription, aberrant mRNA processing, premature translation termination, peptide misfolding, and amino acid misincorporation. Amino acid misincorporation is perhaps the most prevalent cause of protein synthesis errors and is a result of either decoding errors at the ribosome or tRNA

misaminoacylation. To determine the impact of PheRS QC in preventing tRNA misaminoacylation and how this, in turn, effects protein synthesis accuracy, we quantified $tRNA^{Phe}$ cognate and non-cognate aminoacylation in tandem with amino misincorporation rates. A five-fold increase compared to wild type in misaminoacylated tRNA accumulation was observed in the PheRS QC deficient strain subjected to Tyr stress, with Tyr-$tRNA^{Phe}$ accounting for 5.3±0.5% of the cellular aminoacylated $tRNA^{Phe}$ pool. The elevated level of tRNA misaminoacylation observed in the absence of editing correlated well with mistranslation rates under the same conditions, with 7.5±2% of Phe codons being translated as Tyr. Taken together, these data show that proofreading and editing of misaminoacylated Tyr-$tRNA^{Phe}$ by PheRS is the primary QC checkpoint that prevents mistranslation of Phe codons during growth under amino acid stress conditions.

Despite mistranslation of Phe codons rising to over 7% in the absence of PheRS QC, this did not lead to activation of unfolded protein stress responses. Previous studies in in aa-tRNA editing deficient strains of *E. coli* have observed mistranslation rates as high as 10% causing no adverse effects on growth (23). Even under optimal growth conditions, nonsense suppression of stop codons in wild type *B. subtilis* occurs at a rate of 0.4% (24). Overall, organism specific experimental measurements of mistranslation have been observed to range from 0.001-10%, with an estimated 15% of all proteins in the cell possessing at least one misincorporated amino acid. It is estimated that 50% of amino acid misincorporation events affect protein stability, often leading to protein misfolding, yet the impact of specific natural amino acid substitutions on overall proteome stability remains largely unknown (17). Despite comparatively high levels of Tyr misincorporation in the absence of PheRS QC, the protein stress response remained inactive indicating that Phe to Tyr mistranslation is well-tolerated in yeast. Conversely, similar growth

conditions in an *E. coli* strain deficient in Tyr-tRNA^{Phe} editing triggered activation of several protein stress response pathways (17). The divergence in these responses to Tyr misincorporation is reflected by the divergent active site topologies of yeast and bacterial PheRSs. Unlike yeast cytoplasmic PheRS, the bacterial homologues of PheRS are able to prevent Tyr misacylation through high level discrimination within the amino acid binding pocket of the active site. All fungal, and some higher eukaryote, cytosolic PheRS enzymes possess a natural Ala to Gly substitution within the active site that enlarges the amino acid binding pocket sufficiently for near cognate amino acids to be efficiently activated (17). The lower amino acid specificity of yeast PheRS inversely correlates with the organism's ability to tolerate protein stress resulting from Tyr misincorporation. The opposite is true for *E. coli,* whose PheRS is more discriminating and less tolerant of mistranslation (17). Through comparison of these systems, it becomes clear that the level of amino acid misincorporation is less important than the nature of the substitution itself. In yeast, 7% *para*-tyrosine substitution at Phe codons is well-tolerated, yet 1% misincorporation of *meta*-tyrosine in *E. coli* results in activation of protein stress responses and severe growth defects (17). These observations show that the protein error rates vary substantially from system to system and that, beyond the level of error, amino acid specific chemicophysical properties play critical roles in determining the cytotoxicity of mistranslation.

Discussion:

The role of aaRS editing activity has previously been defined in the context of mistranslation. However, until these studies, the specific mutation rate of an editing deficient strain of yeast had not been determined. It was shown that the mutation rate under high Tyr

conditions was two orders of magnitude higher for both the wild type and mutant strains, as compared to minimal media. The editing deficient strain had a mutation rate approximately a half order of magnitude more than the wild type strain, when grown in high Tyr. This result indicated that the high Tyr stress caused mistranslation when PheRS editing activity was deleted. Additional research could be conducted to determine if higher mutation rate would be observed for other similar amino acids, such as leucine and isoleucine.

Furthermore, aaRS mutations are directly linked to human disease either by mistranslation or by disruption of secondary functions of aaRS. mtPheRS mutations were linked to infantile neurologic diseases that led to PheRS structures that were not as functionally capable of accurate translation. However, there are other mutations in aaRS that were shown to cause human disease. Due to the presence of aaRS species throughout evolution, these enzymes likely had multiple roles in earlier organisms. Therefore, this further sheds light on the impact of aaRS mutations that do not affect translation, but rather disrupt cell localization, protein-protein interactions, and binding of different molecules in the cell to induce a response pathway. Thus, aaRS not only have a role in translation, but they are also important in other signaling responses in the cell. Mutations in these multiple roles of aaRS species lead to a variety of different diseases.

In addition, misincorporation of Tyr by PheRS was shown to prevent the activation of the GAAC. When the amount of Phe is low in the cell, Tyr is often incorporated by PheRS and then translated into protein. However, the resulting proteins often are not misfolded enough for the cell to recognize that there was a misincorporation. Thus, if aaRS editing is not functional, then when PheRS binds Tyr instead of its cognate tRNA, the cell is not able to sense an amino acid stress because the misincorporation prevents an accumulation of deacylated aaRS. In other

words, aaRS editing activity is critical to maintaining cellular responses at an appropriate level. Without this editing activity, organisms are much more susceptible to decreased adaptability to changing environments and disease.

References:

1. Bullwinkle, T.J. and Ibba, M. (2016) Translation quality control is critical for bacterial responses to amino acid stress. *Proc Natl Acad Sci USA*, **113**, 2252-2257.
2. Kim, S., You, S., and Hwang, D. (2011) Aminoacyl-tRNA synthetases and tumorigenesis: more than housekeeping. *Nature*, **11**, 708-718.
3. Zhu, Y.O., et al. (2014) Precise estimates of mutation rate and spectrum in yeast. *PNAS*, **111**, 2310-2318.
4. (2014) Improving the performance and analysis of fluctuation assays. 19-38.
5. Luria, S.E. and Delbruck, M. (1943) Mutations of Bacteria from Virus Sensitivity to Virus Resistance. *Genetics*, **28**, 491-511.
6. Storici, F. and Resnick, M.A. (2004) The Delitto Perfetto Approach to In Vivo Site-Directed Mutagenesis and Chromosome Rearrangements with Synthetic Oligonucleotides in Yeast. *Methods in Enzymology*, **409**, 329-345.
7. Guo, M. and Schimmel, P. (2013) Essential Non-Translational Functions of tRNA Synthetases. *Nature Chemical Biology*, **9**, 145-153.
8. Lee, C.W. et al. (2015) Overexpressed typtophanyl-tRNA synthetase, an angiostatic protein, enhances oral cancer invasiveness. *Oncotarget*, **6**, 21979-21992.

9. Sherman, J.M., Rogers, M.J., and Soll, D. (1992) Competition of aminoacyl-tRNA synthetases for tRNA ensures the accuracy of aminoacylation. *Nucleic Acids Research*, **20**, 2847-2852.
10. Konovalova, S. and Tyynismaa, H. (2013) Mitochondrial aminoacyl-tRNA synthetases in human disease. *Mol Genet Metabol*, **108**, 206-211.
11. Walker, M.A., et al. (2016) Novel Compound Heterozygous Mutations Expand the Recognized Phenotypes of FARS2-Linked Disease. *Journal of Child Neurology*, **31,** 1127-1137.
12. Almalki, A., et al. (2014) Mutation of the human mitochondrial phenylalanyl-tRNA synthetase causes infantile-onset epilepsy and cytochrome c oxidase deficiency. *Biochem Biophys Acta*., **1842**, 56-64.
13. Park, S.G., Schimmel, P., and Kim, S. (2008) Aminoacyl tRNA synthetases and their connections to disease. *PNAS*, **105**, 11043-11049.
14. Tamura, K. and Schimmel, P. (2001) Oligonucleotide-directed peptide synthesis in a ribosome- and ribozyme-free system. *Proc Natl Acad Sci USA*, **98**, 1393-1397.
15. Jordanova, A. et al. (2006) Disrupted function and axonal distribution of mutant tyranosy-tRNA synthetase in dominant intermediate Charcot-Marie-Tooth neuropathy. *Nat Genet*, **38**, 197-202.
16. Nangle, L.A., et al. (2007) Charcot-Marie-Tooth disease-associated mutant tRNA synthetases linked to altered dimer interface and neurite distribution defect. *Proc Natl Acad Sci USA*, **104**, 11239-11244.
17. Mohler, K., et al. (2017) Editing of misaminoacylated tRNA controls the sensitivity of amino acid stress responses in Saccharomyces cerevisiae. *Nucleic Acids Research.*

18. Phizicky, E.M. and Hopper, A.K. (2010) tRNA biology charges to the front. *Genes Dev*, **24**, 1832-1860.
19. Kirchner, S. and Ignatova, Z. (2015) Emerging roles of tRNA in adaptive translation, signalling dynamics and disease. *Nat Rev Genet*, **16**, 98-112.
20. Ling, J., Yadavalli, S.S. and Ibba, M. (2007) Phenylalanyl-tRNA synthetase editing defects result in efficient mistranslation of phenylalanine codons as tyrosine. *RNA*, **13**, 1881-1886.
21. Roy, H., Ling, J., Irnov, M. and Ibba, M. (2004) Post-transfer editing in vitro and in vivo by the beta subunit of phenylalanyl-tRNA synthetase. *EMBO J*, **23**, 4639-4648.
22. Narasimhan, J., Staschke, K.A. and Wek, R.C. (2004) Dimerization is required for activation of eIF2 kinase Gcn2 in response to diverse environmental stress conditions. *J Biol Chem*, **279**, 22820-22832.
23. Hauryliuk, V., Atkinson, G.C., Murakami, K.S., Tenson, T. and Gerdes, K. (2015) Recent functional insights into the role of (p)ppGpp in bacterial physiology. *Nature reviews*, **13**, 298-309.
24. Roy, H., Ling, J., Alfonzo, J. and Ibba, M. (2005) Loss of editing activity during the evolution of mitochondrial phenylalanyl-tRNA synthetase. *J Biol Chem*, **280**, 38186-38192.